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The highly conserved, N-terminal $(RXXX)_8$ motif of mouse Shadoo mediates nuclear accumulation

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ABSTRACT

The prion protein (PrP)—known for its central role in transmissible spongiform encephalopathies—has been reported to possess two nuclear localization signals and localize in the nuclei of certain cells in various forms. Although these data are superficially contradictory, it is apparent that nuclear forms of the prion protein can be found in cells in either the healthy or the diseased state. Here we report that Shadoo (Sho)—a member of the prion protein superfamily—is also found in the nucleus of several neural and non-neural cell lines as visualized by using an YFP-Sho construct. This nuclear localization is mediated by the (25-61) fragment of mouse Sho encompassing an $(RXXX)_8$ motif. Bioinformatic analysis shows that the $(RXXX)_n$ motif (n = 7-8) is a highly conserved and characteristic part of mammalian Shadoo proteins. Experiments to assess if Sho enters the nucleus by facilitated transport gave no decisive results: the inhibition of active processes that require energy in the cell, abolishes nuclear but not nucleolar accumulation. However, the $(RXXX)_8$ motif is not able to mediate the nuclear transport of large fusion constructs exceeding the size limit of the nuclear pore for passive entry. Tracing the journey of various forms of Sho from translation to the nucleus and discerning the potential nuclear function of PrP and Sho requires further studies.

1. Introduction

Transmissible spongiform encephalopathies are a group of rare, infectious, lethal neurodegenerative disorders in mammals, including Creutzfeldt–Jakob disease in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep and goat, and chronic wasting disease in mule deer and elk [1,2]. The infectious agent contains an improperly folded form of the host-encoded prion protein (PrP^C). Conversion of PrP^C into the disease-associated isoform, termed PrP^{Sc}, is thought to be the primary pathogenic event, although the mechanisms by which the conversion to PrP^{Sc} causes disease are poorly understood [3]. Deciphering the physiological role of PrP may hold a key to this question; however, despite intensive research over decades, the exact physiological function of the prion protein remains unclear.

PrP is expressed throughout the body, but in varying amounts; the highest levels occur in the central nervous system (CNS) and the heart [4]. It has been alleged to associate with more than fifty interacting partner molecules [5,6] and its involvement in copper homeostasis, neuroprotection and signaling, memory, proliferation, differentiation, apoptosis, myelination, circadian rhythm, cell adhesion, synaptic activity, etc. [5,7,8] has been proposed. However, it is not clear which, if any, of these functions can explain the conserved occurrence of PrP across many species including mammals, birds, fishes and amphibians. To attribute such a function to PrP, Shadoo, the newest member of the prion superfamily may provide some hint [9].

The Shadoo protein (Shadow of Prion Protein, *SPRN* gene), was discovered *in silico* by Premzl et al. in 2003. They noted that the structure of the predicted Shadoo protein loosely resembles the flexibly disordered N-terminal part of the prion protein. In particular there

Abbreviations: aa, amino acid; ATP, adenosine triphosphate; β-Gal, β-Galactosidase; $(Cherry)_2$, tandem Cherry dimer; $(Cherry)_4$, tandem Cherry tetramer; CNS, central nervous system; DEG, 2-deoxy-D-glucose; DMEM, High-glucose Dulbecco's Modified Eagle medium; ECL, enhanced chemiluminescence; ER, endoplasmatic reticulum; FITC-BSA, fluorescein isothiocyanate labelled bovine serum albumin; GFP, enhanced green fluorescent protein; GPI anchor, glycosylphosphatidylinositol anchor; HD, hydrophobic domain; NLS, nuclear localization signal; NRS, nuclear retention signal; PBS, phosphate buffered saline; PrP, prion protein (C: cellular, Sc: scrapie); QKI-5, quaking-I 5 protein; RPMI medium, Roswell Park Memorial Institute Medium; RT, room temperature; Sho, Shadoo protein (shadow of the prion protein); *snls*, short predicted nuclear localization signal; SS, signal sequence (ER-targeting); SV40, simian vacuolating virus 40; TTF-1, thyroid transcription factor 1; YFP, enhanced yellow fluorescent protein

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are two features which are present in both Shadoo's and PrP's natively unstructured N-terminus: i) a series of tandem repeats of short sequences with positively charged residues (from aa. 26 to aa. 48 in mouse Shadoo, termed XXRG tetrarepeats, and from aa. 51 to aa. 90 in mouse PrP); ii) and a hydrophobic domain (HD) (from aa. 63 to aa. 82 in mouse Shadoo and from aa. 111 to aa. 130 in mouse PrP). The predicted subcellular localization of both proteins is the same—i.e. both possess glycophosphatidyl-inositol (GPI) anchor signal sequences and one or two N-glycosylation sites that are suggestive of their release to the secretory pathways—laying further emphasis on their similarity [9,10].

The presence of the GPI anchor and N-glycosylation were experimentally demonstrated on recombinant Sho constructs with or without a tag as well as on the endogenous protein in brain homogenates [11,12].

Besides these structural similarities, several other analogies have been identified between PrP and Sho.

Their expression reaches its highest levels in the CNS [4,9,10,12]. Endoproteolysis close to the center of PrP generates C1 and N1 fragments which likely have important biological properties [13,14]. C-terminal fragment of Sho resembling PrP's C1 is readily detected in brain samples and in the cell-associated fraction from lysed cultured cells [12]. Besides these data, PrP endoproteolysed adjacent to the GPI anchor that releases a nearly full length form of the molecule which can be reproduced *in vitro* by ADAM 10 [15,16]. Likewise, a glycosylated form of Sho is easily detectable within the conditioned medium of Sho expressing cells [17].

Functional analogies have also been confirmed: the toxic effect of the third prion protein superfamily member, Doppel, and internal deletion mutant forms of PrP could be counteracted by the co-expression of either PrP or Sho in transfected granule cell neurons. This rescue effect was abolished by the deletion of the HD region [12]. Tatzelt and co-workers reached to a similar conclusion investigating the rescue effect of the expression of PrP or Sho on kainate or glutamate induced apoptosis in SH-SY5Y cells. These rescue effects could also be abolished by the deletion of the HD region [18,19]. In addition, some experiments on *PRNP*-knockout mice suggest that the two proteins have overlapping functions and can complement each other [20,21], although this issue is rather controversial [22,23].

To further strengthen the argument for the similarity between these two proteins, formation of homodimers of both PrP and Sho have been demonstrated when expressed in cell culture. These associations are facilitated by the HD region in the case of both proteins. Furthermore the formation of heterodimers between PrP and Sho mediated also by the HD region has also been shown [6,18,19,24]. In addition to this protein-protein interaction, more than a dozen proteins outside the prion protein superfamily have been identified that might interact with both PrP and Sho [6,25]. The similarity between their binding partners appears to go beyond protein-protein interactions: nucleic acids have also been shown to bind PrP [26–28] and it has also been proposed that they bind Sho as well [29]. Sho's tetrarepeats include an RGG box motif which is present in RNA-binding proteins involved in various aspects of RNA processing as well as mediating interactions to other proteins [29].

An overlap in the subcellular distribution pattern of minor populations of PrP and Sho, would further buttresses the case for the functional analogy between these two proteins. PrP has been shown to have two short sequences, referred to as NLS1 and NLS2 that, under some conditions, function as nuclear localization signals (NLS) [30,31]. Various forms of PrP, such as PrP^{Sc} [32], truncated PrPs representing prion proteins with familial mutations [33], or PrP expressed without endoplasmic reticulum (ER)-targeting signal sequences and GPI anchor by genetic engineering [34] or by alternative translation initiation [35,36] have been demonstrated to appear in both the cytosol and nucleus. In addition, cellular PrP has been reported in the nucleus of proliferating epithelial cells [37] as well as in endocrine and neuronal cells [38]. In line with these observations, Sho has a potential arginine-methylation site that is a common post-translational modification in RGG-box domains and occurs on proteins that are found in the nucleocytoplasmic compartment [29]. More interestingly, the endogenous Shadoo appears in the cell bodies of some of the hypothalamic neurons and it was found in nuclear fractions of both brain and cultured cells [22].

Here we observed a fluorescent tagged form of Sho in the nucleus of several cell lines, and found nuclear localization signals in the sequence of Sho by *PredictNLS* [39] and by *NLStradamus* [40]. These have prompted us to experimentally validate whether any of these sequences functions as an NLS: which in turn would not only add one more item to the growing list of similarities between PrP and Sho, but might also point to a common function of PrP and Sho in the nucleocytoplasmic compartment.

2. Materials and methods

Chemicals: The chemicals used were purchased from Sigma-Aldrich, unless otherwise stated.

2.1. Plasmid constructions

To generate YFP-, and GFP-chimeric Shadoo constructs, generally the N-terminal NheI and AgeI restriction sites (to insert ER-targeting signals or Shadoo core sequences), and the C-terminal EcoRI and BamHI restriction sites (to insert Shadoo, GPI-anchor signal, potential NLS sequences) were used in pEYFP-C1 and pEGFP-C1 (Clontech) respectively. GFP-β-Gal and NLS^{x3SV40}-GFP-β-Gal were kind gifts of Y. Lazebnik. To generate Sho-GFP-β-Gal and $(25\text{-}61)^{Sho}$-GFP-β-Gal XhoI and XbaI restriction sites were used. More details for plasmid constructions are provided in the supplementary documents (Supp. Table 1). Restriction enzymes were brought from Fermentas. The deletion mutants were generated by Quickchange Mutagenesis (Stratagene). All constructs were verified by sequencing before being used. The synthesis of the oligonucleotides used and the sequencing of the DNA constructs were carried out by Microsynth AG (http://microsynth.ch/).

2.2. Cell culture

Cells (HeLa, SH-SY5Y, Cos-7, Zpl2-1, Zw3-5, Hpl3-4 and Hw13-3) were grown at 37 °C in a humidified atmosphere of 5% CO_2 in high-glucose Dulbecco's Modified Eagle medium (DMEM, Lonza) supplemented with 10% heat inactivated fetal bovine serum (Lonza) and containing 100 units/ml penicillin and 100 μg/ml streptomycin (Lonza).

K562 cells were cultured in Roswell Park Memorial Institute Medium (RPMI)-1640 medium supplemented with 2.0 g/l sodium-bicarbonate, 10% fetal bovine serum (Lonza) and 1% GlutaMAX (Gibco).

Zpl2-1, Zw3-5 [41], Hpl3-4 and Hw13-3 [42] are immortalized, hippocampal cell lines, they were kind gifts of Y. S. Kim and T. Onodera respectively.

2.3. Transfection

Cells were cultured on Labtek II 8 well chambers (Labtek) or 6 well plates (Greiner Bio-One), seeded a day before the transfection at a density of 2×10^4 cells/well or 3×10^5 cells/well respectively. The next day, at around 50% confluence, cells were transiently transfected with plasmid constructs using Turbofect (Fermentas), briefly as follows: DMEM was changed on the cells to fresh one, containing 10% FBS. Typically 500 ng/4000 μg plasmid and 0.5 μl/4 μl Turbofect was mixed in 50 μl/400 μl serum free DMEM and the mixture was incubated for 40 min at RT prior adding to cells. Transfection medium was changed to fresh supplemented DMEM after 3 h of incubation.